The use of bacterial membrane vesicles in vaccines:

Implications for future development of a vaccine against *Mycobacterium tuberculosis*

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Abbreviations

OMV : outer membrane vesicle
MV : membrane vesicle
LPS : lipopolysaccharide
DCs : dendritic cells
TLR-4 : toll-like receptor 4
LPB : LPS binding protein
NO : nitric oxide
ETEC : enterogenic E. coli
IL-10 : interleukin 10
Tregs : regulatory T-cells
IBD : inflammatory bowel disease
PQS : P. aeruginosa quorum-signalling protein
LT : heat-labile enterotoxin
OM : Outer membrane
PG : peptidoglycan
IM : inner membrane
Lpp : Braun's lipoprotein
EDL : electrodense layer
LC : large capsule
SC : small capsule
APC : antigen presenting cell
MHC : major histocompatibility complex
OMP : outer membrane protein
CFA : complete Freund's adjuvant
sOMVs : spontaneous outer membrane vesicles
dOMVs : detergent outer membrane vesicles
nOMVs : native outer membrane vesicles
IT : Intra-tracheal
TNF : tumor necrosis factor
DOC : deoxycholate
NIPH : Norwegian institute for public health
SBA : Serum bactericidal assays
GFP : Green fluorescent protein
TB : tuberculosis
BCG : bacillus Calmette-Guérin
WHO : World health organization
MDR-TB : multidrug-resistant TB
XDR-TB : extensive drug resistant TB

Abstract

Living bacteria produce membrane vesicles during growth, whether in culture or in an infected host. The notion that gram-negative bacteria produce outer membrane vesicles (OMV) has been around for over 40 years however the physiological functions and the biogenesis of OMVs are not fully understood. OMVs are small, non-replicating, vesicles derived from the outer membrane of viable gram-negative bacteria and possess immunogenic properties. OMVs have therefore been used in vaccine development since the late 80's. OMV vaccines are considered a safe and effective prevention method against *Neisseria meningitidis* infections. Despite the lack of an outer membrane, gram-positive bacteria were found to produce membrane vesicles (MVs). The knowledge of gram-positive MVs is limited and the possible application of such MVs in vaccines remains to be elucidated. This thesis provides an overview of the field of OMV vaccines and a possible strategy for the use of MVs in a vaccine against *mycobacterium tuberculosis*.

Bacteria produce membrane vesicles

Bacteria need to interact with their environment to ensure growth and survival. Secretion of proteins in MVs is one of the available means of bacterial communication. The production of MVs by gram-negative bacteria such as *Neisseria meningitidis* and *Escherichia coli* was first observed over 40 years ago, however, more recently gram-positive bacteria including myco-bacteria were also found to produce MVs [1 2]. Currently it is widely accepted that all bacteria produce MVs [1 2 3 4]. Bacterial vesicles are produced by growing cell cultures whether grown in liquid culture, solid culture or biofilms and the release of MVs by living bacteria is shown to be distinct from membrane blebbing found during cell lysis or cell death [5 6 7 1]. The mechanistic details behind MV production are not fully elucidated however there have been various observations that led to a general consensus that MV production is a highly-conserved pathway among all bacteria. There are currently no known bacterial strains that lack the ability to produce MVs and no growth conditions or genetic modifications are known to cause full inhibition of MV production [8]. Production of MVs leads to a loss of lipids and proteins that need to be replaced hence the production of MVs is an energy sink. It could therefore be suggested that it is unlikely that bacterial MV production does not have an important conserved function for bacteria. Furthermore it has been observed that certain proteins can be enriched or depleted in MVs resulting in different physiological benefits for the parent-strain [8]. Proteomics studies comparing two separate batches of OMVs isolated from culture media of the same bacterial strain reveals an overlap of nearly 75% of all proteins however, some proteins abundant in one batch were almost absent in the other batch. The enrichment and exclusion of certain proteins observed in proteomics of MVs suggests active regulation of the constituents by a sorting mechanism during MV production. This is in line with the idea that MV production is an active process and that

perhaps bacteria produce different MVs with different physiological purposes [33].

Physiological functions of MVs

Bacteria produce MVs containing biologically active proteins that perform diverse biological processes to improve survival chances and enhance growth. Using MVs to transport cargo allows bacteria to secrete and transport soluble as well as membrane bound molecules over a long distance. The membrane of MVs further shields and protects bacterial factors. Currently MVs are considered to play a crucial role in bacterial stress response, intracellular communication, biofilm formation and pathogenesis [8]. OMVs contain a large amount of endotoxin better known as lipopolysacharide (LPS), derived from the outer membrane of gram-negative bacteria [31]. LPS is a danger signal for the infected human host and leads to the production of pro-inflammatory cytokines by immune cells such as macrophages and dendritic cells (DCs). Upon infection with a gram-negative bacteria, toll-like receptor 4 (TLR-4) in the CD14+ T-cells of the host will recognize LPS bound to the LPS binding protein (LPB) in complex with an adaptor protein called MD2 and cause production of pro-inflammatory cytokines and nitric oxide (NO) [10 11]. Inflammation is an important part of the innate immune system, however, in some cases a severe response to LPS may lead to septic shock and death [12]. Production of LPS-containing OMVs by gram-negative bacteria is therefore commonly associated with damaging effects to the infected host [6]. In general pathogenic strains of bacteria produce more OMVs [13]. For example, enterogenic *E. coli* (ETEC) was shown to produce roughly 10 fold more vesicles than non-pathogenic *E. coli* and leukotoxic *Actinobacillus actinomycetemcomitans* produces over 25 times more OMVs than its non-leukotoxic variant [14 15]. The increased production of MVs by pathogenic

gram-negative bacteria combined with the knowledge that bacterial MVs can be detected in fluids of infected hosts led to the hypothesis that pathogenic bacteria release MVs to disperse virulence factors that increases their rates of survival and spreading in the infected host [16]. Whether the same increase in MV production is also found in pathogenic strains of gram-positive bacteria is uncertain. However, proteomic studies comparing the pathogenic *M. tuberculosis* and *M. bovis BCG* with the non-pathogenic *M. smegmatis* reveal that there was an enrichment of lipoproteins in MVs from *M. tuberculosis* and *M. bovis* which was absent in *M. smegmatis* [1]. The enrichment in lipoproteins could hint at increased MV release found in the pathogenic strains however no quantitative data about MV production was disclosed in these experiments [1]. The difference in constituents of MVs produced by different mycobacteria could suggest that bacteria actively regulate constituents of MVs depending on the physiological aim of MV production. Interestingly, experiments have shown that mice pre-treated with *M. bovis* derived MVs via intra-tracheal injection show more acute inflammation and a higher bacterial load after receiving an aerosol challenge with the parent strain compared to mice that received MVs from the non-pathogenic *M. smegmatis* [1]. This observation shows that MVs can increase virulence of pathogenic bacteria.

Bacterial production of MVs is not always only beneficial for the bacteria. There are examples of mutual benefits resulting from inter-kingdom communication between microbiota and mammals with the use of MVs [17]. For example, *B. fragilis* uses OMVs to communicate with dendritic cells (DCs) of the host [28]. The OMVs internalized by the DCs contain polysaccharide A, causing increased secretion of Interleukin-10 (IL-10) by DCs. IL-10 leads to an increased production of regulatory T-cells (Tregs) causing immunological tolerance to the bacteria thereby preventing the development of inflammatory bowel disease (IBD). Interestingly DCs treated with OMVs from a PSA knockout strain of *B. fragilis* did not show suppressed pro-inflammatory cytokine production and were unable to

prevent wasting disease in mice [28]. PSA is therefore suggested to have therapeutic potential as a treatment option for immunologic disease such as IBD or MS. However, it could be the case that purified PSA alone is unable to provoke a DC response since earlier experiments evaluating the response of epithelial cells to heat-labile enterotoxin (LT) have shown that cells may respond differently to the same substrate depending on whether it is presented in MVs or as a soluble factor [18].

Another physiological role for MV production, clearly beneficial to the bacteria, is aimed at creating a niche for growth and survival. One way to secure an optimal environment would be bacterial use of MVs as an offensive tactic against other bacteria in their direct surroundings. Protease and toxin-containing vesicles from various bacterial strains can interact via fusion or an adherence mechanism with other gram-positive or gram-negative bacteria which can cause lysis of the targeted bacteria [19] [20]. OMVs produced by *Pseudomonas aeruginosa* contain peptidoglycan hydrolases. These OMVs can associate with gram-positive bacteria due to charge-interactions or fuse with the membrane of other gram-negative bacteria triggering lysis of the recipient [21]. Destruction of competing strains may be beneficial in creating a niche for growth and survival. Examples of exchange of beneficial material between bacteria using MVs have also been described. Native *P. aeruginosa* vesicles are for example able to transfer antibiotic-resistance enzymes to other bacteria to increase survival chances of the recipient [22]. Creating a habitable niche is also realized by the formation of a biofilm. Biofilms are a cluster of bacteria embedded in an extracellular matrix that is composed largely of polysaccharides produced by the bacteria. OMVs are shown to contribute 52% of the LPS present in the bio-film matrix and could make up 16,8% of the total matrix material [25] . Biofilm can be formed on many surfaces including inside the invaded host [23]. The formation of biofilm creates a protected living environment for bacteria as the inner layers may be out of reach of the hosts defence and antibiotics. Antibiotic resistance can be

increased a 1000-fold in bacteria living in a biofilm compared to planktonic bacteria from the same strain [24] making treatment of the bacterial infection challenging. OMVs are suggested to play a role in the protection against the host and increase antibiotic resistance by aiding in biofilm formation. Biofilm formation could therefore be considered another virulent factor of OMVs.

More ways of creating antibiotic resistance have been described which reveals OMV production as a stress-response. For example, increased OMV production was observed in *P. aeruginosa* upon treatment with a β-lactam antibiotic. Interestingly these OMVs were shown to contain enzymes called β-lactamase, these enzymes are needed for the degradation of β-lactam. Using OMVs to secrete β-lactamase enable the bacteria to defend themselves against treatment with β-lactam antibiotics [89].

Lastly, a physiological function for which OMVs may also be used is nutrient acquisition. OMVs from *P. aeruginosa* contain the *P. aeruginosa* quorum-signalling protein (PQS). PQS is a hydrophobic protein able to bind iron. Bacteria produce OMVs containing PQS that function as iron scavengers. It has been suggested that after release OMVs can scavenge iron from the environment. They may then re-fuse with the outer membrane to secure supply of sufficient amounts of iron, which is a known limiting factor for bacteria [26].

At present, most knowledge of the functions of bacterial MVs is based on observations and experiments done with gram-negative bacteria. Figure 1 gives a schematic overview of the physiological interactions involving OMVs derived from gram-negative bacteria.

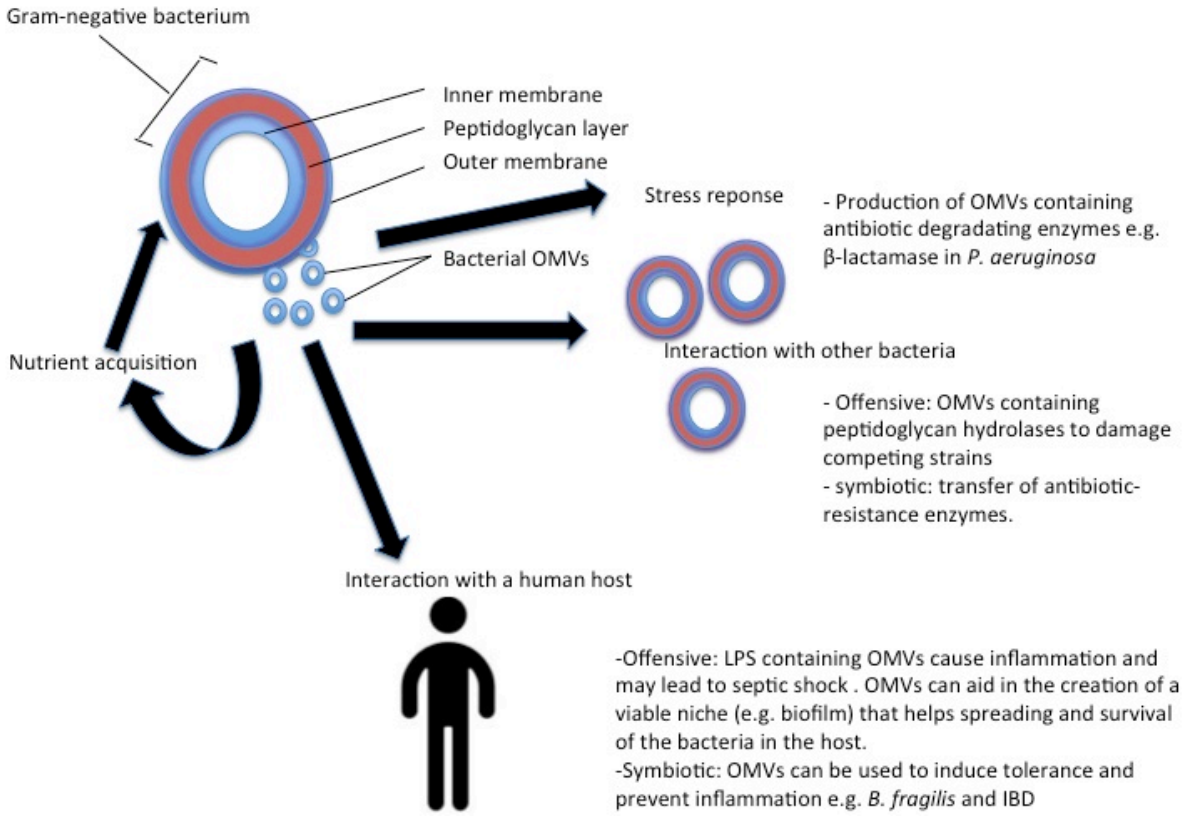

Figure 1. Physiological interactions engaging OMVs derived from gram-negative bacteria.

Biogenesis of MVs

All bacteria are known to produce MVs and although decades of research has been dedicated to elucidate why bacteria produce MVs, the mechanisms behind MV production are less well studied and remain to be elucidated [6]. Especially in gram-positive bacteria this process is only just beginning to be explored [2]. Based on proteomic data is has been proposed that OMVs produced by gram-negative bacteria are derived from the outer membrane of the bacteria as was revealed by proteomic analysis. Typically OMVs are bi-layered spheroid structures with a size

ranging from 20 to 250 nm in diameter [8]. Gram-positive bacteria have been shown to produce MVs which appear as bi-layered spherical structures ranging from 20 to 130 nm in diameter [2 29]. However, also sizes more similar to the gram-negative OMVs have been observed [52]. Considering the fact that the cell wall architecture differs between gram-positive and gram-negative bacteria (Fig. 2) it is likely that the mechanisms behind MV production differ as well.

In order to assess how bacteria may form MVs it is important to know how the bacterial cell wall is structured. The cell wall of gram-negative bacteria consists of three compartments: the outer-membrane (OM) the peptidoglycan layer (PG) and the inner membrane (IM) (fig 2a). The three layers are well interconnected by proteins such as Braun's lipoprotein (Lpp), OmpA and lppAB to maintain cell rigidity and to protect the contents of the cell. The outer membrane is an asymmetric bi-layer of which the outer leaflet contains mainly LPS. LPS consists of lipid A, core polysaccharide and O antigen. The inner leaflet of the outer membrane consists of phospholipids. The leaflets of the inner membrane consist of phospholipids that form a bi-layer [31 36]. The gram-negative derived bacterial OMVs consist mainly of the outer membrane, however proteins derived from the cytoplasm of the bacteria are also shown to consistently end up in OMVs [32 33]. This observation hints at a mechanism able to regulate the cargo and release in OMVs. Gram-positive bacteria have a thick cell wall consisting mainly of a large peptidoglycan layer that surrounds the cytoplasmic membrane. The peptidoglycan layer allows for diffusion of metabolites to the plasma membrane and may protect the bacteria in hostile environments such as those encountered in a host species. Without this peptidoglycan layer gram-positive bacteria would be lysed due to the osmotic pressure differences across the cell wall. The cytoplasmic membrane consists of a phospholipid bi-layer (fig 2b).

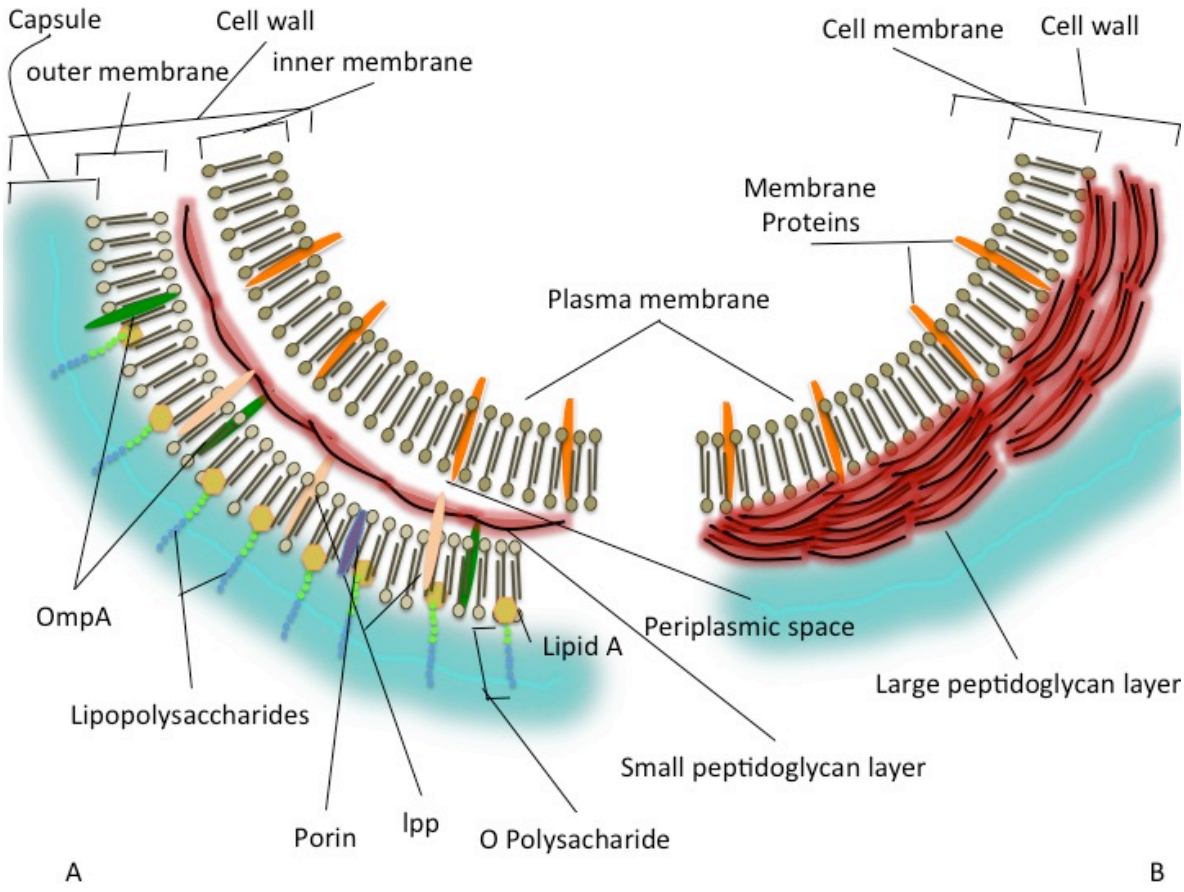

Figure 2. The structure of the bacterial cell wall.

The cell wall of a gram-negative bacteria consisting of an inner and outer membrane linked by a thin peptidoglycan layer with lypoproteins such as OmpA and Lpp. The outer membrane contains porins and LPS (A). The gram-positive cell wall consists of a single phospholipid bi-layer and a large peptidoglycan layer (B). Both types of bacteria may additionally have a bacterial capsule (A,B).

The degree to which bacterial cells produce MVs may also vary depending on the bacterial capsule (fig 2) [34]. The bacterial capsule is a layer on the outside of the cell wall that usually consists of polysaccharides but may also contain polypeptides [30 58]. Gram-positive as well as gram-negative bacteria can have a bacterial capsule however they are not found in all species [35]. The bacterial capsule layer can be subdivided in at least 3 subtypes; a large capsule (LC), a small capsule (SC) and an electron dense layer (EDL) [34]. Using density gradient centrifugation to isolate the various capsule-subtypes of cultured *Bacteroides fragilis* led to the notion that bacterial OMVs were produced at a much higher rate by bacteria with

an EDL as compared to bacteria from the same strain of the LC subtype [34]. In a heterogeneous culture of *B. fragilis* the EDL subtype covers about 5% of total cells. This finding suggests that most OMVs produced by a colony of *B. fragilis* are derived mainly from this small portion of the total bacteria. Microscopic images suggest that OMVs may get stuck in the large capsule hindering vesicle release [34]. Whether this difference in amounts of MVs produced by bacteria depending on the capsule is universal for all strains with a capsule is unknown.

Formation of MVs by bacteria is likely to be an essential and conserved mechanism as all known bacterial species have been found to produce MVs and no mutations were found to abolish MV production. In gram-negative bacteria formation of OMVs is suggested to start with bulging of the outer membrane at a location where the proteins linking the OM and the PG layer are absent or disorganized [8]. This absence of proteins linking the OM and the PG layer can for example be the result of a physiological process such as cell division but can also be induced by antibiotics or autolysins [38] (Figure 3 A). Bacteria in which the linking lipoproteins are mutated do show hyper-vesiculation [37] and OMV production is observed to be higher in rapidly dividing cultures which is in line with this suggested mechanism [38]. This mechanism does however not account for an increase in OMV secretion caused by an overexpression of misfolded periplasmic proteins and it does not explain the enrichment of certain proteins in the OMVs. Another suggested mechanism is that possibly proteins may accumulate at the inner surface of the plasma membrane where they would produce outward pressure that would result in budding of an OMV only when the OM-PG links are removed at that location. This mechanism would explain the increase in OMV production caused by both accumulation of proteins and removal of OM-PG links and it would also allow gathering of specific proteins resulting in enrichment of such proteins in the OMV[39] (Figure 3 B). Another possible mechanism that may contribute to OMV biogenesis could be an increased presence of

curvature-inducing molecules near the OM that could result in bulging of the membrane after disrupting the OM-PG links (figure 3 C). Although the suggested mechanisms for OMV production seem plausible there is no conclusive evidence for one of these mechanisms yet. More research is needed for elucidation of the process of OMV biogenesis.

Little is known about the biogenesis of MVs by gram-positive bacteria. Based on the heterology of the cell wall it is likely that the process does not resemble OMV production by gram-negative bacteria. Unlike OMVs, MVs are derived from the cytosolic membrane and contain cytosolic proteins. However, the physiological functions reported for MVs and OMVs are similar and enrichment or exclusion of specific proteins suggests that a sorting mechanism for MV production also exists in gram-positive bacteria [2]. It was suggested that production of MVs is the result of over-production of membrane-bound proteins or enzymes. Switching from glucose to starch limitation when culturing *Thermoanaerobacterium thermosulfurogenes* EM1 caused increased production of membrane-bound starch-degrading enzymes and was associated with increased production of MVs [82]. A lack of space to incorporate the newly synthesized enzymes could be a possible explanation. Future research on gram-positive bacterial MV production is needed to elucidate the mechanisms of MV biogenesis.

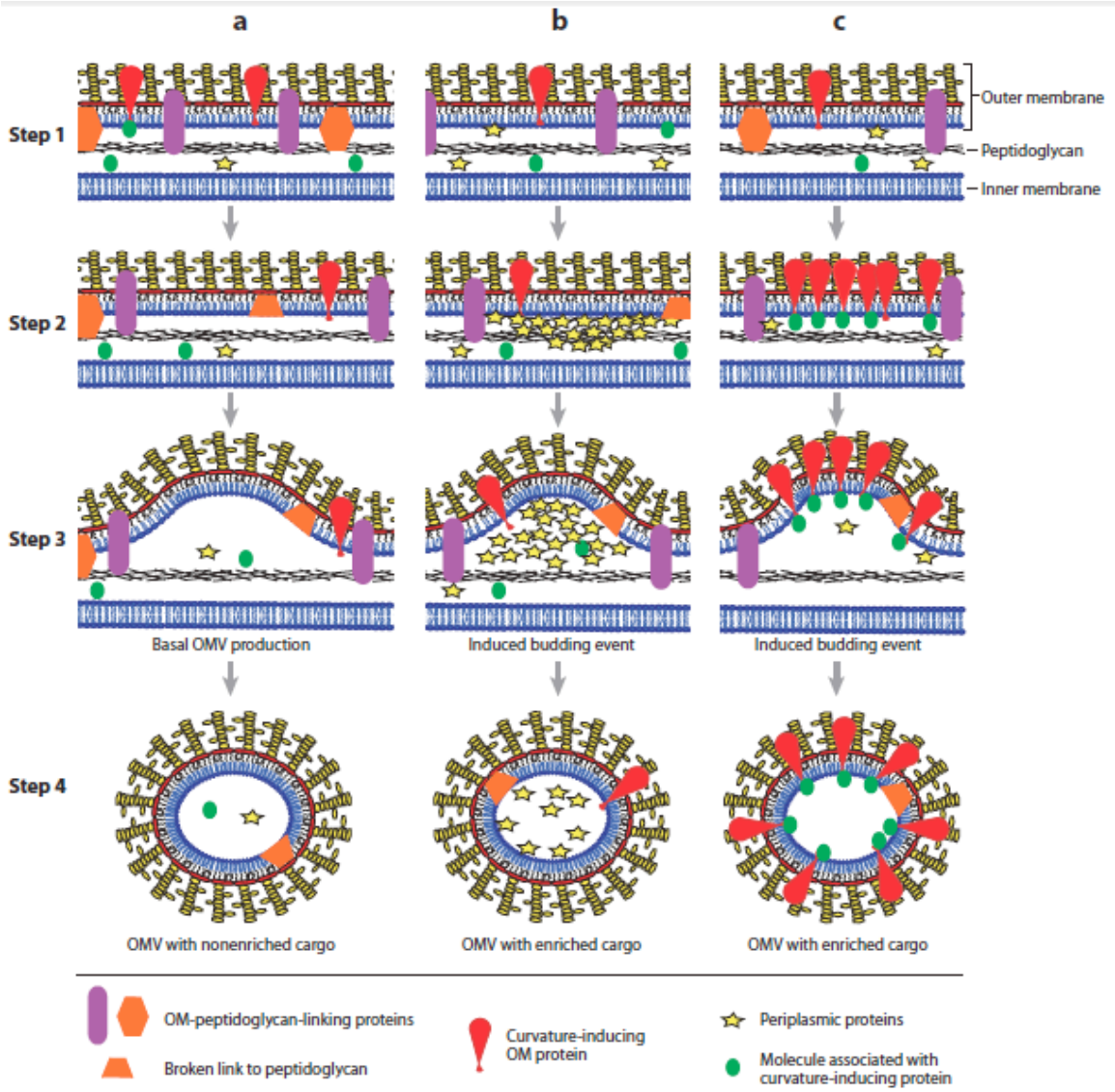

Figure 3. Suggested mechanisms of bacterial OMV production. OMV formation as a result from bulging-out of the outer membrane when lipoproteins linking the OM with the PG are removed or reorganized (a). OMV formation resulting from a combination of pressure on the outer membrane caused by protein aggregates and removed or reorganized lipoproteins (b). OMV production as a result of the increased presence of curvature-inducing proteins disrupting the bond between OM and PG (c). Adapted from Kulp *et al.* 2010 [8]

The role of OMVs in vaccine development

According to the world health organization's health report of 2004 an estimated 25% of deaths in 2002 were the result of infectious disease [83]. The use of vaccines to induce immunization has greatly reduced the mortality associated with a variety of infectious diseases and remains to be the most effective and efficient method to combat infectious pathogens. To induce adaptive immunity professional antigen-presenting cells (APC), especially dendritic cells, should present antigens derived from a pathogen on their cell surface in the context of major histocompatibility complex (MHC) molecules. Naïve T-cells specific for the antigen-MHC complex become activated and can help naïve B-cells specific for the pathogen to differentiate into plasma-B-cells that are able to produce pathogen specific antibodies or can kill infected cells. There are several strategies to produce a vaccine able to induce adaptive immunity against a bacterial pathogen. Vaccines can for example be based on i) attenuated weakened bacteria or killed bacteria, ii) a (synthetic) protein subunit from bacteria, iii) a conjugate of capsular polysaccharides with a protein or iv) MVs.

All strategies have their own advantages and drawbacks. In general the use of whole living bacteria is a good way to trigger and "teach" the immune system. However vaccines based on attenuated live bacteria are not always safe because of the risk of adverse effects in immune compromised patients, such as HIV-patients, or the risk that attenuated bacteria may mutate and revert to a virulent form [43]. Using protein sub-units may solve this health-risk problem. However bacterial species may evolve or mutate in such a way that the antigens in the subunit vaccine no longer lead to recognition of the bacterial pathogen. Furthermore because of their lower antigenicity the use of protein subunit vaccines requires multiple immunizations or the addition of adjuvants [47] which leads to a decrease in the ease of use and an increase in costs. This is

especially be problematic in developing countries where access to healthcare is cumbersome. In some cases, such as with *Neisseria meningitidis* conjugate vaccines were successfully developed against various subtypes [48]. However, the development of a glycoconjugate vaccine against *N. meningitidis* sero-group B was less successful which was suggested to be the result of similarity between the conjugate and part of a human glycoprotein [49]. In the case of *N. meningitidis* sero-group B bacterial OMV vaccines were able to solve this problem [50]. Bacterial MVs containing a selected set of bacterial proteins may serve as antigens recognized by the immune system. These immunogenic properties combined with the fact that OMVs derived from gram-negative bacteria have self-adjuvant properties makes OMVs an interesting target for vaccine development [40]. In the last decades development of vaccines using bacterial MVs has gained a lot of attention [41] [42] [43]. As OMVs are derived from the outer membrane of gram-negative bacteria they contain outer membrane proteins (OMPs). OMPs, especially in combination with the LPS present in OMVs, can be potent antigens. OMV vaccines derived from a single strain of wildtype bacteria can induce a sufficient immune response. Examples of such vaccines are the MenB and the MenzB OMV vaccines against meningococcal infection caused by *N. meningitidis* sero-group B in Norway and New Zealand [53] [63] [62]. Although OMVs derived from *N. meningitidis* sero-group B where able to induce immunity, the immune response was found to be mainly directed against a single antigen and was therefore not able to protect against all subtypes of *N. meningitidis* sero-group B [51].

Producing an OMV vaccine from a single isolated strain may therefore result in poor coverage of immunization in a population due to variations in the immunogenic membrane proteins in subtypes of the strain.

In conclusion, the use of OMVs in vaccine development is an established method in producing vaccines against *N. meningitidis* and might hold potential to be used in development of vaccines against other bacterial pathogens.

Little research has been done on the possibilities of using MVs from gram-positive pathogens in vaccine development [52]. Experiments done with MVs isolated from culture supernatant of *Bacillus anthracis* show that intraperitoneal injections of MVs in mice combined with complete Freund's adjuvant (CFA) can protect mice from lethal *B. anthracis* infections [52]. Mice treated with MVs also produced antibody responses after receiving purified toxins that were also present in the MVs as determined with ELISA [52]. Similar experiments were done in mice using intra-tracheal (IT) administration of MVs derived from *M. tuberculosis*. Bone marrow derived macrophages incubated with MVs isolated from *M. tuberculosis* respond with the production of inflammatory cytokines such as IL-6, IL-10 and tumor necrosis factor (TNF). Mice pre-treated with *M. tuberculosis* MVs showed a higher bacterial load and increased inflammation after aerosol infection with the parental strain as compared to control group mice that received PBS or MVs from a non-pathogenic bacterial strain [1]. Knockout experiments revealed that this inflammatory response was TLR2-dependent. These results suggest that *M. tuberculosis* derived MVs are a detrimental factor during a tuberculosis infection however other methods of administrating *M. tuberculosis* MVs may cause a different response and more research is needed to analyze the potential of MVs in vaccine development.

Isolation of OMVs

MV isolation processes can be divided into two categories, detergent extraction and detergent-free extraction and different isolation processes yield different MVs [60](fig. 4). Production of OMVs starts with culturing bacteria. After a period of expansion the bacteria are pelleted from the medium by centrifugation at around 2,900xg. The supernatant can be

filtered and spontaneous OMVs (sOMVs) can be isolated with ultra centrifugation (fig. 4). The cell pellet is re-suspended in a sodium chloride buffer. After homogenization cells are pelleted re-suspended in a tris-buffer containing EDTA. EDTA is used to decrease the stability of the bacterial outer-membrane and hence increases OMV production [55]. After incubation in the EDTA buffer, deoxycholate (DOC) detergent is added to remove LPS from the mixture of cells and OMVs. OMVs are then separated from the cell debris using 20000xg centrifugation. The pellet is discarded and a filtration step using a 0.2μM filter can be used to remove protein aggragates and cell debris. MVs are then pelleted from the supernatant with ultra centrifugation. The OMVs are resuspended in an EDTA buffer containing a small amount of DOC to remove the last LPS after which the detergent OMVs (dOMVs) are pelleted and resuspended in a 3% sucrose buffer (fig. 4) [54]. Alternatively OMVs can be isolated without the addition of DOC to the cells or the OMV buffer yielding native OMVs (nOMVs) that maintain the LPS (fig.4).

Gram-positive MVs can be isolated in a similar fashion to the OMVs. The cells are pelleted and MVs are isolated with a series of centrifugation and filtration to exclude aggregates and cell debris before eventually isolationg OMVs with ultracentrifugation at 100000xg for one hour [1].

During the isolation of OMVs from gram-negative bacteria for vaccine development purposes, DOC is added to decrease the amount of LPS in OMVs, as too much LPS causes a severe inflammatory response that can be associated with fever and coagulation [57]. However, LPS is also needed as an adjuvant [59]. Another downside of the use of detergent is that, other than LPS, also phospholipids and lipoproteins that could contribute to immunogenicity might be removed from the OMVs [58]. It could therefore be argued that using detergent-free isolation methods may yield OMVs with better immunogenic properties however LPS toxicity would be an issue. The removal of LPS by DOC also causes aggregation of OMVs, which may thwart filter sterilization making the use of possibly toxic preservatives necessary [60]. Using detergent-free isolation prevents

aggregation and may therefor simplify the production of OMV vaccines by removing the detergent and the preservation steps from the process. Experiments comparing the composition of dOMVs, nOMVs and sOMVs by looking at five major biochemical compounds of OMV vaccines revealed that the use of DOC leads to selective removal of LPS and phospholipids [60]. These OMVs were isolated from trivalent PorA *N. meningitides* strain with lpxL1 mutation. As a result of LPS and phospholipids removal the dOMVs contained an increased fraction of PorA and other proteins which suggests that they may be able to supply more antigens when used in a vaccine. Interestingly, experiments in mice comparing the immunogenic properties of the various OMVs revealed that nOMVs and sOMVs cause improved cross-protection compared to dOMVs while having comparable toxicity [60]. Similar results were found when comparing sOMVs and dOMVs from an *N. meningitidis* strain without the lpxL mutation however, toxicity was not analyzed [69]. In both cases the increased immunogenicity may result from the increased presence of non-protein immunogenic antigens such as the phospholipids. Another possibility is that improved adjuvant activity resulting from increased presence of LPS led to the increase in cross-protection. These possibilities are not mutually exclusive so these observations may result from a combination of the two suggested explanations [70].

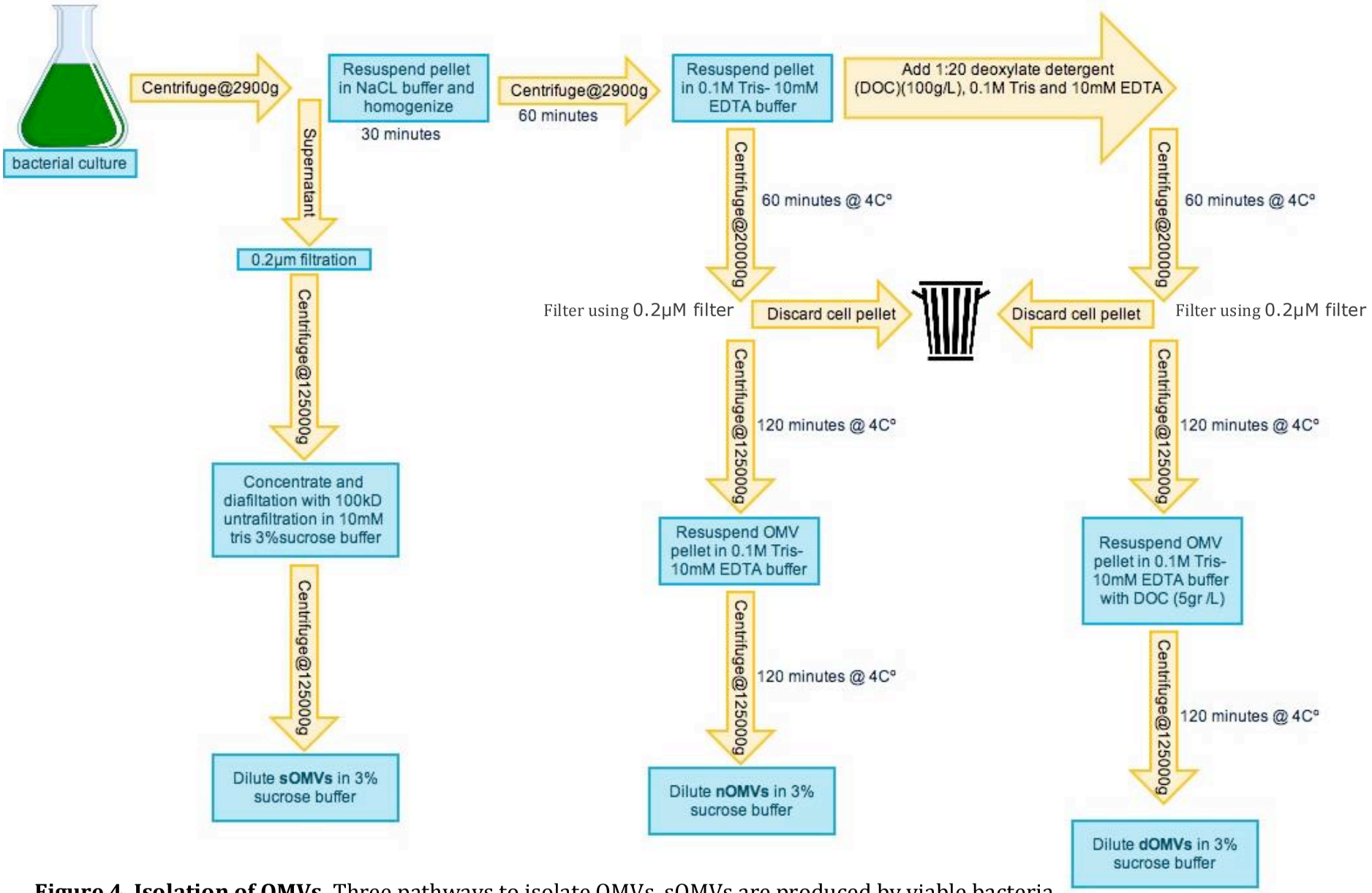

Figure 4. Isolation of OMVs. Three pathways to isolate OMVs. sOMVs are produced by viable bacteria and can be isolated from the culture media bacteria are grown in. nOMVs are similar to sOMVs as they maintain LPS however, EDTA treatment increases yield and changes protein constituents.. dOMVs are nearly deplete of LPS and may be less antigenic due to a loss of proteins caused by DOC treatment.

Production of OMV vaccines

When the proof-of-concept for the use of OMV-vaccines arose in the late 1980's, the Norwegian institute for public health (NIPH) developed a production process for OMV vaccines that was used to prevent outbreaks in various countries [53]. The first step in preparing OMV-based vaccines is the culture of bacteria that were isolated from an infected host to create a "tailor-made" vaccine strain based on a current outbreak [54]. OMVs used for vaccine production were originally dOMVs[56]. In order to prepare the vaccine from the isolated dOMV the OMVs can be purified using a discontinuous sucrose gradient combined with ultracentrifugation [84] however not all reports specify this step [54]. Additionally an adjuvant, such as aluminium hydroxide or aluminium phosphate, can be added to the vaccine [53 54]. A preservative, such as thiomersalate, can be added to the final product [60]. The successfully used MeNZB OMV vaccine against meningococcal serotype B infection, contained per vaccin-dose 25 µg OMV-proteins isolated from the strain causing the epidemic [61]. In this vaccine aluminium hydroxide was added as an adjuvant to boost the immune response to the antigenic outer membrane proteins (OMPs) present in the OMVs. NaCl was added to modify the tonicity of the vaccine, histidine was used to buffer the pH and sterile water was used as a diluent for injection [61 62].

OMV vaccine engineering

The process of transforming a bacterial culture into a functional batch of OMV vaccines is elaborate and several steps have been reconsidered over the years as the field of OMV vaccine research progressed. Discovery of a variety of genetic mutations has enabled the improvement of OMV vaccine development in multiple ways. In the early years of OMV vaccine development OMVs were commonly produced from a bacterial strain

isolated from an infected host during an epidemic[50 85]. These vaccines were effective for the outbreak at that time. However, they were not suitable for global use due to variability in PorA[50 85]. PorA is a class 1 outer membrane protein found in *N. meningitidis* that is known to be an immuno-dominant antigen. The genetic variety of PorA in wild-type *N. meningitidis* sero-group B strains made it difficult to produce a vaccine able to induce immunization against the various subtypes using either the conjugate OMP-subunit strategy or OMVs from a single wild-type strain [64 56]. To overcome this problem and produce a more universally applicable *N. meningitidis* sero-group B vaccine, researchers have used genetically modified stains containing three subtypes of PorA, so called trivalent strains. OMVs isolated from two trivalent strains were used to create a hexavalent vaccine with a protein content constituting for >90% of the various porA OMPs [65]. OMVs isolated from a third trivalent strain were added later to include immunogenicity to even more subtypes of PorA in a vaccine called NonaMen [66]. The goal of producing a multivalent vaccine is to create a universal vaccine protective against the various subtypes of *N. meningitidis*. Serum bactericidal assays (SBA) measuring functional antibodies against MenB revealed that combining OMVs from different strains containing multiple PorA subtypes in a vaccine led to production of antibodies against all nine PorA subtypes in mice [66]. Using such a vaccine can decrease the amount of vaccinations needed to gain functional immunity against multiple subtypes. Further experiments revealed that combining the NonaMen vaccine with other vaccinations that are currently part of the childhood vaccination programs such as the DTaP/IPV–Hib vaccine caused no significant decrease in its functionality [66]. Combining vaccines can increase the ease of use of vaccines and would allow for more immunizations at the same time thereby boosting the immune response and making them more cost-effective. Including more antigens and improving their immunogenicity is one reason to use genetically modified bacteria. However, these are not the only beneficial modifications. In some cases it is beneficial to remove certain OMPs from

the bacteria. An example is the removal of the B-polysaccharide and lacto-N-tetraose from *Neisseria meningitidis* [54]. These structures are homologous in humans and hence may cause either a poor immune response or an auto immune response that could be detrimental [54].
An example of a genetic modification that has shifted the focus from dOMVs to nOMS and the use detergent-free OMV vaccines is the inactivation of the lpxL gene. The lpxL gene regulates acyloxyacylation of lipid A and inactivation of this gene led to formation of LPS in which lipid A was penta-acylated whereas it is normally hexa-acylated [67]. This lpxL mutation causes attenuated LPS toxicity while the adjuvant activity is maintained and may therefore be a solution for the use of detergent-free OMVs in vaccine development [67]. Another bottleneck of using detergent free isolation methods in vaccine development is the significantly lower yield [60]. A low yield would especially be problematic in large-scale production. Mutations in the rmpM gene had been hypothesized to lead to increased OMV production, as the rmpM protein is a suggested anchoring protein that causes an interaction between the outer membrane and the peptidoglycan layer of gram-negative bacteria [71]. Removal of the rmpM gene causes a phenotype with a loosely attached outer membrane and leads to a significant increased OMV production in *N. meningitidis* [60]. As nOMVs and sOMVs contain more immunogenic properties perhaps smaller and fewer doses may be needed to induce sufficient immunization in the recipient in which case this would compensate at least partly for the lower yield. Large-scale production of OMV vaccines that use nOMVs or sOMVs is not currently part of clinical practice; these findings are a step in the right direction for the so-called next-generation OMV vaccines.
Interestingly, recent discoveries reveal that the use of OMVs does not necessarily have to be limited to their use in vaccines against their parent strain. Experiments have shown that OMVs from non-pathogenic bacteria can be used as an antigen-presenting vehicle [72]. Proteomic studies of *Escherichia coli* derived OMVs have shown that the ClyA protein is consistently enriched in OMVs [72 86]. It was suggested that fusion of

heterologous proteins to ClyA would result in inclusion of the fused protein in secreted OMVs. Perhaps this could lead to antigen-presentation of such a heterologous protein that would have increased immunogenic effects compared to injection with the purified antigen itself. In-vivo experiments in mice have shown that treatment with *Escherichia coli* OMVs derived from a mutant strain containing green-fluorescent protein (GFP) fused with ClyA, resulted in antibody titers that were not found when immunizing with purified GFP [72]. Using this principle it should theoretically be possible to produce a safe vaccine based on OMVs against virtually every pathogen.

Future prospects for a vaccine against *M. tuberculosis*

Tuberculosis (TB) is an infectious disease caused by *M. tuberculosis* that mostly affects the lungs of patients. TB is an airborne infection that is spread by coughing or exhaling by people with an active TB infection. After inhalation the pathogen is phagocytized by macrophages residing in the lung. *M. tuberculosis* may survive in macrophages as it is able to inhibit phagosome maturation and prevent fusion with a lysosome [73]. The currently available bacillus Calmette-Guérin (BCG) vaccine is based on attenuated live bacteria from the *M. bovis* strain however this vaccine has unreliable protection against pulmonary tuberculosis in the adult population [74]. Around two billion people in the world are infected with *M. tuberculosis* however about 90% of infected people has a latent infection and show no clinical signs. According to the WHO, TB is second only to HIV/AIDS when it comes to causing death by a single infectious agent. The risk of developing active TB for patients with a latent infection is around 10% however this percentage is increased when patients have a weakened immune system such as HIV patients. If active TB is left untreated it causes death in about 66% of the cases. Drug-sensitive TB can be cured with a standard six-month course of four antimicrobial

drugs. This form of treatment has been used for decades and multidrug-resistant TB (MDR-TB) has evolved. MDR-TB does not respond to at least two of the most powerful first-line anti-TB drugs and patients require second-line TB-drugs and extensive chemotherapy. An even more drug resistant TB infection exist, extensive drug resistant TB (XDR-TB) which does not responds to most available TB medicines. Due to this increase in drug-resistance and the poor protective capacity of the BCG vaccine development of a new and effective vaccine against TB has been an on-going aim of research in the last decades [74].

Various options to produce a new TB-vaccine or improve the existing BCG vaccine are currently under investigation and many are in clinical trials. Vaccines that are currently in the pipeline are mostly based on protein-subunits combined with adjuvants or viral vectors and some on inactivated live bacteria from other mycobacterial strains [75]. Interestingly no MV based vaccines are currently in clinical trials and little experimental data about *M. tuberculosis* MVs is published.

M. tuberculosis is able to prevent antigen presentation by macrophages as is can decrease the expression of MHC class ll transactivator and MHC class ll molecules. Another main bottlenecks in TB vaccine development is lack of knowledge of immunodominant antigens. It is suggested that the currently used BCG vaccine does not express immunogenic antigens sufficiently and therefore mutant strains containing suggested antigenic proteins are being developed to create an improved BCG vaccine [76]. Using "reversed vaccinology", the genome of *M. tuberculosis* was analyzed to find epitopes that may aid in inducing a sufficient immune response. Elucidating what proteins may have immunogenic properties could be valuable for vaccine development [77].

Using the knowledge of the field of OMV vaccines and the recent developments of OMV vaccine engineering it would be interesting to

explore the possibilities of the development of a TB vaccine based on MVs or perhaps OMVs from a non-pathogenic gram-negative bacterial strain containing antigenic material derived from *M. tuberculosis.* Apoptotic vesicles from *M. tuberculosis* infected macrophages have already been shown to induce a specific CD8+ T-cell response against *M. tuberculosis* as a result of MHC class I antigen presentation after being engulfed by DCs [78]. These apoptotic vesicles may not resemble MVs isolated from M. tuberculosis however, it would be interesting to see whether engulfment of MVs by DCs would also lead to antigen presentation and a CD8+ T-cell response.

IT injection of MVs derived from *M. tuberculosis* did not lead to clinical benefits in mice that were afterwards infected with *M. tuberculosis* via and aerosol challenge [1]. It could be that other ways of administering such MVs or administering these MVs to another species could lead to better results and more research is needed to map the potential of spontaneous *M. tuberculosis MVs*. A relatively simple strategy for development of an *M. tuberculosis* vaccine would therefore be simple injection of MVs combined with an established adjuvant. (fig. 5 option 1)

Fusion of newly discovered antigens with ClyA in *E. Coli* may lead to production of OMVs with antigenicity against *M. tuberculosis.* In order to obtain better coverage to a variety of *M. tuberculosis* sub-types, one may create various mutant strains of *E. coli* expression different antigens and combine the OMVs in the final vaccine. Attenuating LPS toxicity while maintaining LPS adjuvant activity can be realized by using a lpxM mutant strain [79]. Mutations in the OmpA may lead to an increased production of OMVs as this gene was shown to be involved in membrane stability and is partly homologous with the rmpM gene in *N. meningitides* [80] [81]. Detergent free isolation would be used to simplify the production and sterilizing process and both sOMVs and nOMVs could possibly be used in combination. If necessary an adjuvant such as aluminum hydroxide may

be added however the pressense of attenuated LPS may supply sufficient adjuvant activity. This suggested method is just an example and other methods using MVs or engineered OMVs may hold possibilities. The main benefits why using engineered OMVs instead of MVs could be beneficial is the fact that OMVs are well established as relatively easy to produce, self adjuvating, non replicating "antigen vehicles" that can be used in vaccines. It has already been shown that with fusion of genes antigenic proteins can be inserted into OMVs [72]. It would be interesting to combine the knowledge of the established OMV vaccine field with the newly emerging information of possible *M. tuberculosis* antigens derived from the field immunoinformatics[88] and the recently discovered ClyA fusion technique in such a "hybrid vaccine" (fig 5 option 2).

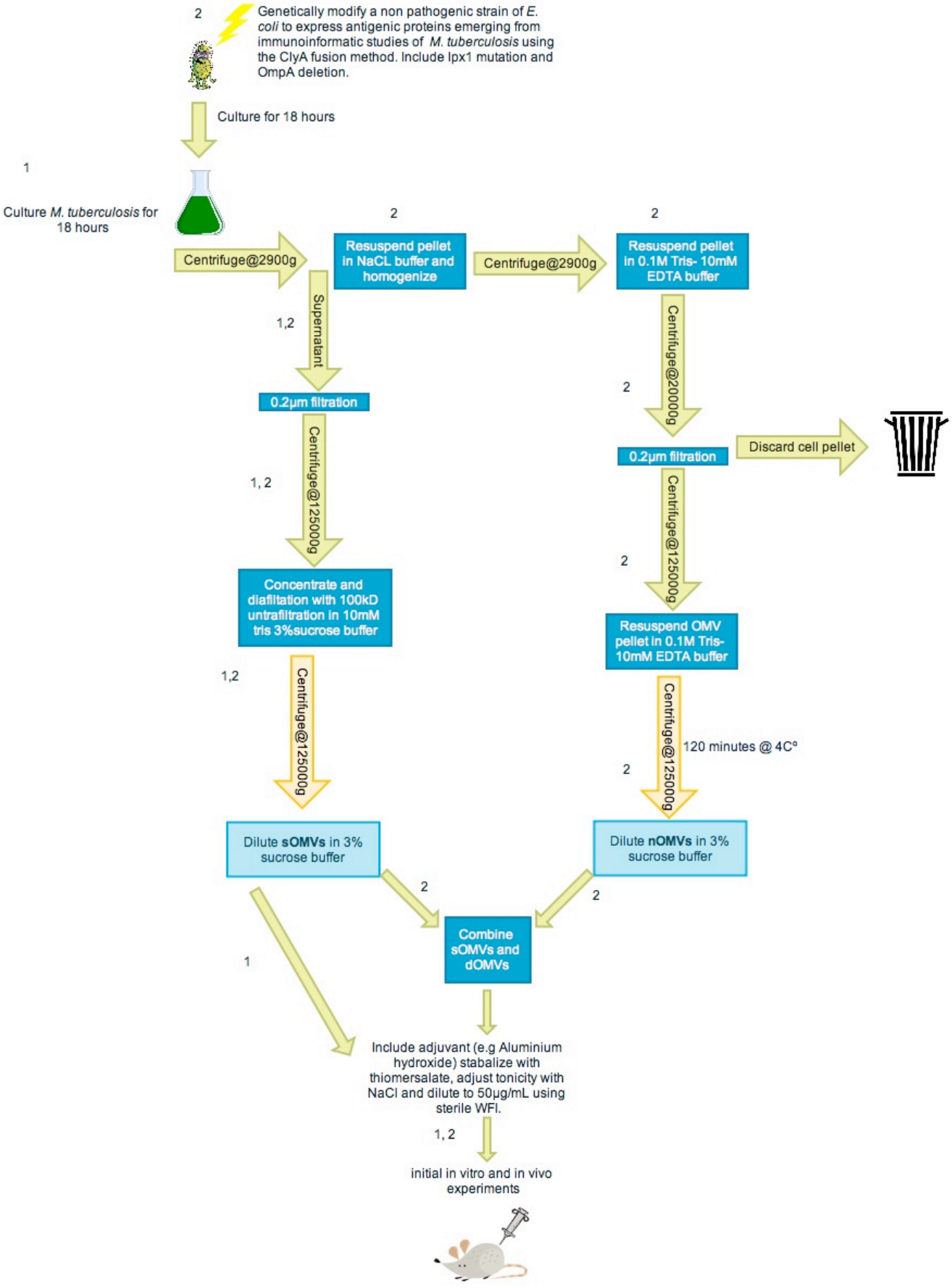

Figure 5. Schematic representation of the development of two suggested (O)MV Mtb vaccines. Option 1 uses MVs isolated directly from the supernatant of *M. Tuberculosis* cultures. Combined with an established adjuvant the MVs may be able to induce an antigen-specific immune response. Option two combines the field of OMV vaccines with genetic engineering to decrease LPS toxicity (lpx1), possibly increase yield (OmpA deletion) and the ClyA fusion method to produce sOMVs and dOMVs with antigenic properties against *M. tuberculosis.* This method relies on the field of immunoinformatics for the supply of immunodominant proteins of *M.tuberculosis.*

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